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Inhibition of constitutive TNF production is associated with PACAP-mediated differentiation in PC12 cells

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ABSTRACT

The pituitary adenylate cyclase-activating polypeptide (PACAP) is a trophic neuropeptide that promotes cell survival and neuritogenesis in the central and peripheral nervous system. Our previous transcriptomic studies revealed the down-regulation of the cytokine tumor necrosis factor (TNF) during PACAP-induced PC12 cell differentiation. Here we show that TNF is constitutively expressed in PC12 cells in a manner dependent on NF-κB transcription factor, and that PACAP rapidly inhibits TNF expression and secretion. The inhibition occurs through suppression of RelB subunit of NF-κB, and is likely to prevent the deleterious effects of the cytokine on survival and neurite outgrowth during PC12 cell differentiation.

1. Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a 38 amino-acid, α-amidated neuropeptide which exerts various trophic effects in the central and peripheral nervous system, and in peripheral tissues [1,2]. The various actions of PACAP are mediated by two types of G-protein-coupled receptors: a PACAP selective receptor, named PAC1-R, and two PACAP/vasoactive intestinal polypeptide mutual receptors, named VPAC1-R and VPAC2-R [2]. These receptors activate different signal transduction pathways, which in turn regulate transcription of genes associated with cell survival and differentiation, e.g., growth arrest and neurite outgrowth in many neuronal cell types [1]. PACAP has been shown to induce differentiation of PC12 cells towards a neuronal-like phenotype via PAC1 receptor [3,4], thus offering a paradigm to study the signaling cascades leading to neuro-endocrine cell differentiation induced by a ligand of G protein-coupled receptors.

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine which exerts its biological effects through activation of two types of receptors, type I (TNF-R1) and type II (TNF-R2) TNF receptors. At variance with TNF-R2, TNF-R1 possesses a death domain on its intracellular region which confers to TNF the ability to stimulate caspase activity and apoptotic cell death [5]. In brain, TNF has been shown to be involved in different types of injuries and pathologies [6], and to modulate survival, proliferation and differentiation of neuronal cells [7,8]. The effects of the cytokine seem to be dependent on the cell type and the expression of the TNF receptor subtypes [8]. In immune cells, PACAP inhibits production of many pro-inflammatory cytokines including TNF [9]. However, little is known about TNF involvement in PACAP-induced neuronal or neuro-endocrine differentiation. In the present study, we showed that PACAP inhibits TNF gene expression and secretion in order to promote cell survival and neuritogenesis during PC12 cell differentiation.

2. Materials and methods

2.1. Drugs

PACAP38 was synthesized by the solid phase methodology as previously described [4]. PS-1145 was obtained from

Abbreviations: PACAP, pituitary adenylate cyclase-activating polypeptide; TNF, tumor necrosis factor; IKK, inhibitor of NF-κB kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TNF-R, TNF receptor

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Sigma–Aldrich (Saint-Quentin Fallavier, France) and human recombinant TNF was purchased from Eurobio (Courtaboeuf, France).

2.2. Cell culture

Rat pheochromocytoma PC12 cells were obtained from the European Collection of Cell Culture (ECACC, Salisbury, Wiltshire, UK) and maintained in Dulbecco's modified Eagle medium (Sigma–Aldrich), supplemented with 10% horse serum (Invitrogen, Cergy Pontoise, France), 5% fetal bovine serum (Sigma–Aldrich), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 °C in 5% CO_2 humidified atmosphere. The medium was renewed every 2–3 days. Twenty-four h after plating, differentiation of PC12 cells was initiated by adding PACAP38 (100 nM).

2.3. Animals

C57bl/6J embryos were collected at E16 and fixed in 4% PFA overnight. On the next day they were rinsed once in PBS and then soaked for 48 h in 30% sucrose for cryoprotection prior to embedding in OCT and storage at −80 °C. 15 μm-thick transverse sections were cut with a cryostat, mounted on Superfrost + slides, and then stored at −80 °C.

Adult Wistar rats were sacrificed by CO_2 inhalation in order to collect the adrenal glands and the brain, which were immediately frozen on dry ice for RNA extraction.

2.4. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from PC12 cells, rat adrenal gland and rat brain using the Tri-Reagent (Sigma–Aldrich). Approximately 1 μg of total RNA was reverse-transcribed using the ImProm-II™ Reverse Transcription System (Promega, Charbonnières, France). One tenth of the reaction mixture was used to quantify TNF and RelB expression by real-time PCR. Gene-specific forward and reverse primers were designed using the Primer Express software (Applied Biosystems) as follows: 5′-GGCTGCCCCGACTATGTG-3′ and 5′-TGACTTTCTCCTGGTATGAAATGG-3′ for TNF; 5′-GAACACCGTGTG-TAACTGCC-3′ and 5′-ATTCCTTCACCCTCCACCTC-3′ for TNF-R1; 5′-GATGAGAAATCCCAGGATGCAGTAGG-3′ and 5′-TGCTACAGACGTT-CACGATGCAGG-3′ for TNF-R2; 5′-CCTGTCTACGACAAGAAGGACA-3′ and 5′-TGTGCACGTCAGCTTGAGAA-3′ for RelB and, 5′-AACTCCCT-CAAGATTGTCAGCAA-3′ and 5′-TGGTCATGAGCCCTTCCA-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as a reference gene. Real-time PCR was carried out in SYBR Green® PCR Master Mix in the presence of 300 nM specific primers, using an ABI Prism 7000 apparatus (Applied Biosystems, Norwalk, CT). The relative efficiency of the amplification of each gene was assessed, and the amounts of each mRNA were determined by normalizing against GAPDH mRNA levels using the comparative Ct method as described by the apparatus manufacturer (Applied Biosystems). For regular PCR, a 3-μL sample of the RT reaction was then amplified with ReddyMix PCR Master Mix (AB Gene, UK) using the appropriate primers in the GeneAmp PCR System 9700 (Applied Biosystems), in the following conditions: 94 °C, 1 min; 30 cycles of 94 °C, 30 s; 52 °C, 45 s; 68 °C, 1 min; 68 °C, and finally 5 min at 68 °C. The PCR products were resolved on a 1.5% agarose gel containing 0.0001% ethidium bromide.

2.5. Cell viability assay

Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, PC12 cells cultured in 100 μL of medium in 96-well culture plates were incubated with 10 μL of phosphate-buffered saline (PBS) containing 5 mg/ml MTT for 4 h at 37 °C in 5% CO_2 atmosphere. Then, 100 μL of a solubilization solution consisting of 50% N,N-dimethylformamide and 20% sodium dodecyl sulfate (SDS), adjusted to pH 4.7 with acetic acid, was added to the culture medium, and the plates were incubated at 37 °C overnight. The absorbance was then recorded using a microplate reader Bio-tek FL 600 (Bio-tek, Illkirsh, France). Specific optical density (ΔOD570-750) for the MTT product was obtained by subtracting the OD value at 750 nm from that at 570 nm, reflecting the number of viable cells.

2.6. Neurite outgrowth measurement

PC12 cells cultured on coverslips in 6-well culture plates were washed twice with ice-cold PBS, fixed in paraformaldehyde solution (4% w/v) for 15 min, then permeabilized with Triton X-100 solution (0.1% Triton X-100 in PBS) for 20 min. After a blocking step of 1 h with 1 mg/ml BSA in PBS, cells were incubated overnight at 4 °C with primary antibody directed against alpha-tubulin (Tebubio; Le Perray en Yvelines, France). Immunodetection was performed with FITC-conjugated goat anti-mouse IgG (1:300 dilution). After mounting, fluorescence was analyzed using a Leica TCS SP2 confocal microscope (Leica Microsystems, Reuil-Malmaison, France). Neurite analysis was performed using ImageJ software (National Institutes of Health, USA). Neurite outgrowth was evaluated by calculating the number of processes per cell and measuring neurite length. Neurite was defined as a cell process >10 μm.

2.7. SiRNA transfection

PC12 cells were transfected with siRNA (Qiagen, Courtaboeuf, France) by using the Amaxa Nucleofector system and the Cell Line Nucleofector kit V electroporation method (Lonza, Basel, Switzerland) according to the manufacturer's protocol (program U-029). To assess the efficiency of siRNA-induced knockdown, mRNA and

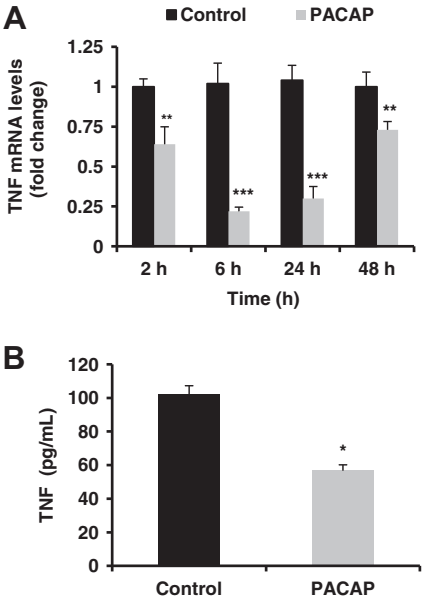

Fig. 1. Effect of PACAP on TNF expression and release. (A) PC12 cells were treated with PACAP (100 nM) for the indicated times, and TNF mRNA levels were quantified by real-time PCR. Data are expressed as means ± SEM relative to control values ($n = 6$). $^{**}p < 0.01$; $^{***}p < 0.001$ (Mann–Whitney U test). (B) PC12 cells were treated with PACAP (100 nM) or left untreated for 6 h. After treatment, the culture medium was collected and TNF was measured in each condition. Data are expressed as means ± SEM relative to control values ($n = 4$). $^{*}p < 0.05$ (Mann–Whitney U test).

protein levels of the target genes were determined by real-time quantitative PCR or Western blot.

2.8. Western blot analysis

PC12 cells cultured in 6-well plates (10^5 cells/well) were lysed in a buffer containing 10 mM Tris–HCl, pH 7.4, 0.05% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation (12,000*g*, 15 min), the supernatant was precipitated with 10% trichloroacetic acid and the whole protein precipitate was washed by ethanol/ether (v/v), and dissolved in electrophoresis-denaturing buffer. Proteins were quantified by the Bradford assay (Bio-Rad, Marnes-la-Coquette, France) to ensure loading of equivalent amounts from all samples. Extracts were separated by SDS–polyacrylamide gel electrophoresis followed by electrotransfer onto nitrocellulose membranes (Amersham Pharmacia Biotech, Orsay, France). RelB was detected using a polyclonal antibody (1:1000 dilution) obtained from Santa Cruz Biotechnology (Heidelberg, Germany), followed by horseradish peroxidase-conjugated secondary antibody (1:5000). The antigen–antibody complexes were visualized by the chemiluminescence ECL Western blotting analysis system (Amersham Pharmacia Biotech) and ChemiDoc XRS + Imaging System (Bio-Rad).

2.9. Immunohistochemistry

After a saturation step of 45 min in 1% goat normal serum, diluted in a solution containing 1% BSA and 0.3% Triton X-100 in PBS at room temperature, mouse sections were incubated overnight with RelB and tyrosine hydroxylase antibodies (1:200) at 4 °C. RelB antibody was obtained from Santa Cruz Biotechnology (Heidelberg, Germany) and tyrosine hydroxylase antibody was obtained from Merck Millipore (Guyancourt, France). These primary antibodies were revealed with secondary anti-rabbit and anti-mouse IgG Alexa Fluor® 488 and 594 (1:300) (Invitrogen). Slides were counterstained with 1 μg/mL 4,6-diamino-2-phenylindole (DAPI, Sigma–Aldrich) in PBS for 1 min. Negative controls were performed using secondary antirabbit and antimouse IgG (1:300) without primary antibodies. In both cases, no immunoreaction was observed. Tissue sections were examined with a Leica SP2 inverted confocal laser scanning microscope (Leica).

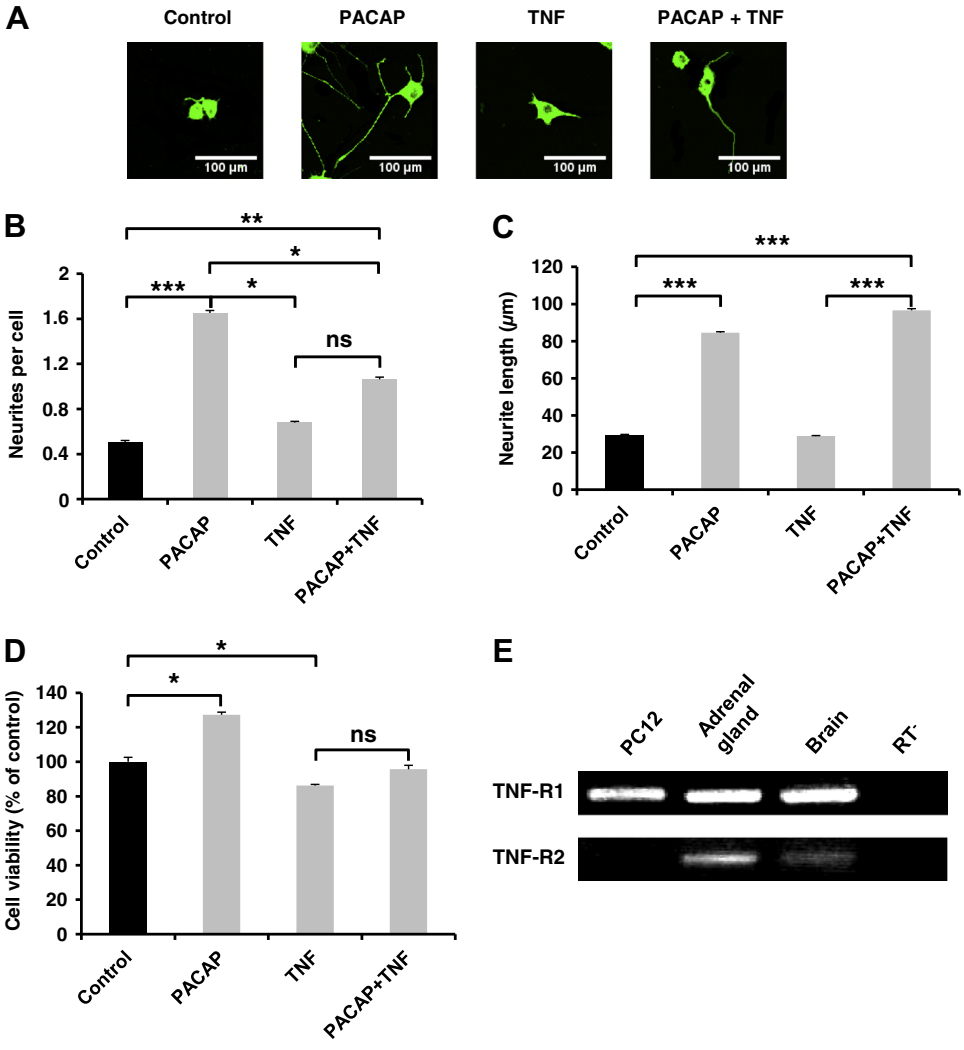

Fig. 2. Effect of TNF on PACAP-induced differentiation in PC12 cells. (A–D) PC12 cells were exposed to PACAP (100 nM) in the presence or absence of TNF (10 μM) for 48 h. Cell morphology changes were analyzed by immunofluorescence confocal microscopy, through labeling of α-tubulin (A). The average number of neurite per cell (B) and neurite length (C) were determined using confocal macroscopy and ImageJ software. Data are expressed as means ± SEM relative to control values. ns, not significant; $^{*}p < 0.05$;$^{**}p < 0.01$; $^{***}p < 0.001$ (Student's *t*-test). Cell viability was assessed using the MTT assay (D). Data are expressed as means ± SEM relative to control values ($n = 6$). ns, not significant; $^{*}p < 0.05$; (Mann–Whitney U test). (E) Total RNA from PC12 cells, rat adrenal gland and brain were reverse transcribed in the presence or absence (RT-) of reverse transcriptase and used to amplify by PCR cDNA fragments of TNF-R1 and TNF-R2.

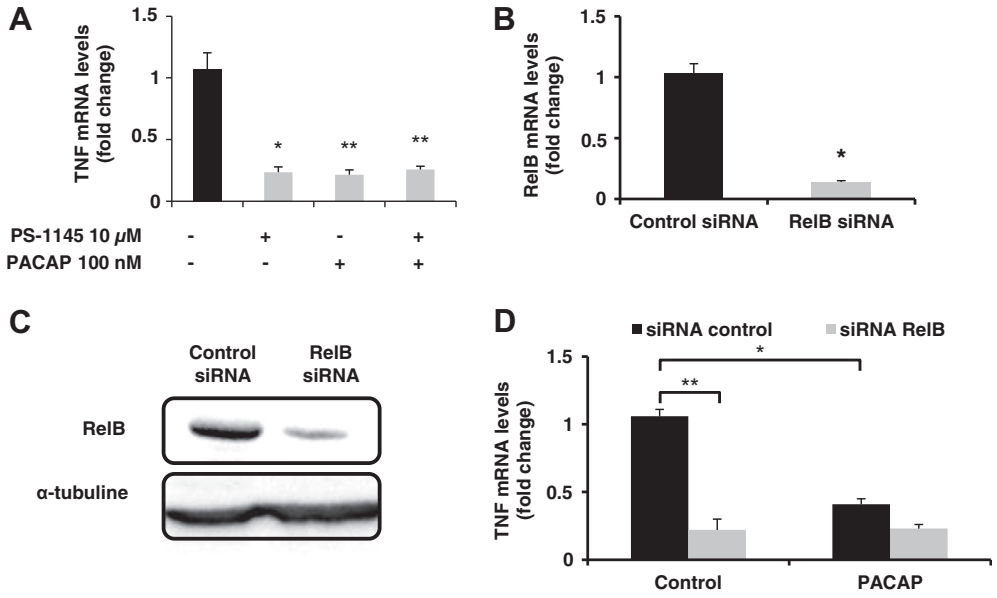

Fig. 3. Role of NF-κB in PACAP-induced TNF inhibition. (A) PC12 cells were treated with PACAP (100 nM) in the presence or absence of PS-1145 (10 μM) for 24 h. TNF gene expression was quantified by real-time PCR. Data are expressed as mean ± SEM relative to control values ($n = 4$). $^{*}p < 0.05$; $^{**}p < 0.01$ (Mann–Whitney U test). (B) Forty-eight hours after transfection of PC12 cells with control or RelB siRNA, RelB gene expression was quantified by real-time PCR. Data are expressed as mean ± SEM relative to control values ($n = 4$). $^{*}p < 0.05$; (Mann–Whitney U test). (C) Western blotting of RelB after siRNA treatment. α-Tubulin was used as a loading control. (D) Twenty-four hours after transfection of PC12 cells with control or RelB siRNA, cells were treated with PACAP (100 nM) for 6 h and TNF mRNA levels were quantified by real-time PCR. Data are expressed as means ± SEM relative to control values ($n = 4$). $^{*}p < 0.05$; $^{**}p < 0.01$; (Mann–Whitney U test).

2.10. Statistical analysis

Results are expressed as means ± standard error of the mean (SEM). Statistical significance was assessed through the non-parametric Mann–Whitney test or Student's *t*-test. A p value < 0.05 was considered statistically significant.

3. Results

3.1. PACAP inhibits TNF production

As a first approach to determine the involvement of TNF in the PACAP effect in PC12 cells, we determined by quantitative PCR analysis the mRNA levels of the cytokine after treatment with the neuropeptide for different times. PACAP rapidly and strongly inhibited the expression of the cytokine; the effects being maximal after 6 h of treatment (Fig. 1A). To assess whether the release of TNF protein could be affected by PACAP, we used a specific ELISA to determine the amounts of the cytokine present in the culture medium after PACAP treatment. This experiment showed that PACAP also induced a 40% decrease in TNF secretion (Fig. 1B). These data suggest that TNF is constitutively expressed in PC12 cells, and that inhibition of its gene expression and its secretion could be involved in the PACAP-mediated effects on differentiation of these cells.

3.2. TNF abolishes the differentiating effects of PACAP through TNF-R1

Microscopic examination revealed that the PACAP-induced morphological changes in PC12 cells are impaired in the presence of TNF (Fig. 2A). Thus, TNF significantly decreased the number of neurites per cell compared to the effect of PACAP alone (Fig. 2B), but did not affect neurite length (Fig. 2C). The MTT assay revealed that the PACAP-elicited increase in cell viability is abolished by co-treatment with TNF (10 μM) (Fig. 2D). In order to determine which TNF receptor is expressed in PC12 cells and could therefore be involved in TNF action in these cells, TNF-R1 and TNF-R2 expression was examined by PCR. This assay revealed that PC12 cells express TNF-R1 but not TNF-R2, while both receptors are present in the rat adrenal gland and brain used as positive controls (Fig. 2E).

3.3. PACAP-inhibited TNF expression involves the NF-κB pathway

Many reports have linked TNF expression to the NF-κB signaling pathway [10,11]. Therefore, we evaluated the effect of PS-1145, a specific blocker of inhibitor of NF-κB kinase (IKK), the kinase responsible for NF-κB activation, on TNF gene expression in PACAP-treated or untreated PC12 cells. In basal condition, we observed an approximately 75% decrease in TNF gene expression in cells treated with PS-1145 (10 μM) (Fig. 3A), suggesting that the NF-κB pathway participates in constitutive expression of the TNF gene in PC12 cells. PACAP inhibited TNF gene expression to levels comparable to those observed with PS-1145 (Fig. 3A). As PACAP and PS-1145 exert similar effects on TNF mRNA levels and the action of the neuropeptide is not modulated by PS-1145 (Fig. 3A), it can be inferred that PACAP-evoked inhibition of TNF could be mediated by suppression of NF-κB activity.

As we showed in a previous study that the nuclear translocation of RelB subunit of NF-κB is reduced by PACAP in PC12 cells [12], we sought to determine whether this NF-κB subunit could be involved in the regulation of TNF gene expression by the neuropeptide. We first determined that treatment with a specific siRNA leads to 86% decrease in RelB mRNA levels in PC12 cells (Fig. 3B). The efficiency of siRNA-induced knockdown of RelB gene expression was confirmed by analysis of the target protein by Western blot analysis (Fig. 3C). In these conditions, we showed that RelB inhibition significantly reduces TNF gene expression in unstimulated cells, and that PACAP has no further effect on TNF gene expression in cells transfected with RelB siRNA (Fig. 3D). These data indicate that RelB may be involved in the regulation of the constitutive expression of TNF and that PACAP inhibits its action.

3.4. RelB is expressed in developing sympathetic neurons

Because the PC12 cell line is considered as a model of sympathetic neuroblasts, we sought to determine if RelB is also expressed

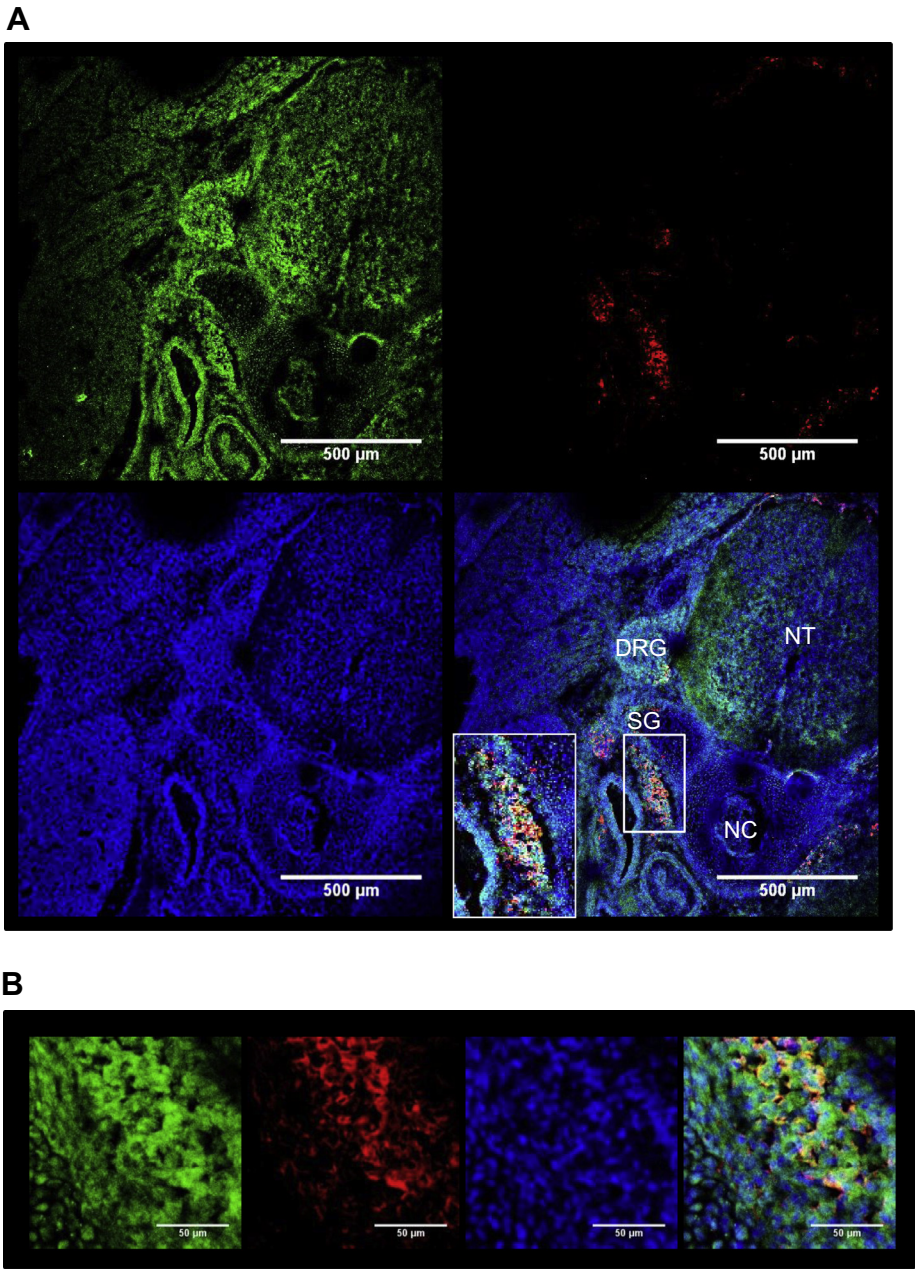

Fig. 4. Expression of RelB in mouse developing sympathetic ganglia. (A) Immunofluorescence of RelB (green), tyrosine hydroxylase (red) and DAPI (blue) in transverse sections from E16 mouse embryos. RelB is widely expressed in all structures of the embryos including sympathetic ganglia, which are co-stained with tyrosine hydroxylase (shown in the box). SG, sympathetic ganglia; DRG, dorsal root ganglia; NC, notochord; NT, neural tube. (B) Sections taken from other ganglia stained for RelB (green), tyrosine hydroxylase (red) and DAPI (blue).

in vivo in developing sympathetic neurons. For this, RelB expression was analyzed by immunohistochemistry in sympathetic precursors of E16 mice embryonic paravertebral sympathetic ganglia (Fig. 4). RelB is indeed expressed in many structures in mouse embryos, including sympathetic neuroblasts which were co-labeled by an antibody directed against tyrosine hydroxylase (Fig. 4). Thus, RelB could play a similar role in sympathetic neuroblasts as in PC12 cells.

4. Discussion

The present findings stem from our initial observation that the expression of the pro-inflammatory cytokine TNF is strongly reduced during PACAP-induced PC12 cell differentiation. This effect implied that the cytokine is constitutively expressed in PC12 cells and that PACAP reduces this expression to be able to exert its trophic action. PACAP not only inhibited TNF gene expression, but also significantly reduced TNF secretion from PC12 cells, indicating that the cytokine is indeed constitutively secreted in basal condition and that PACAP blocks this secretion. Therefore, the cytokine produced by the sympathetic-like cells could act in a paracrine and/or autocrine fashion as it has been shown in microglial cells [13].

In the central and peripheral neurons as in the PC12 cell line, PACAP exerts a protective activity by inhibiting apoptosis and increasing cell viability [14,15], as also observed in the present

study. We found that TNF treatment alters both neuroprotection and neuritogenesis induced by PACAP. This observation correlates with in vivo studies showing that TNF treatment impairs neuronal differentiation [16]. Among the two TNF receptors, we showed that PC12 cells only express TNF-R1 receptor. This receptor, which is expressed in the developing nervous system [17], possesses a death domain allowing the induction of caspase activity and apoptotic cell death upon TNF treatment in different cell types including PC12 cells [5,18] and our unpublished results]. The fact that TNF induces caspase activity confirms that the cytokine interferes with cell survival in PC12 cells as suggested by the MTT assay. Overall, these observations suggest that constitutive expression and secretion of TNF and its action through TNF-R1 could impede the pro-differentiation function of PACAP in neuronal cells. Thus, the inhibition of the cytokine by PACAP could represent a prerequisite for the neuroprotective and differentiating effects of the neuropeptide.

As TNF expression has been shown to be regulated by NF-κB transcription factor in macrophages [19,20], we used PS-1145, an inhibitor of NF-κB signaling, in order to determine if this transcription factor is also able to regulate TNF expression in naive or PACAP-treated PC12 cells. We found that NF-κB activity is necessary for TNF constitutive expression. The inhibition of NF-κB signaling had no further effect on the PACAP-triggered reduction of TNF gene expression. In fact, TNF mRNA levels were similar in PACAP- and PS-1145-treated cells, strongly suggesting that PACAP regulates TNF expression by inhibiting NF-κB activity. Among the five subunits of NF-κB, we have previously shown that PACAP inhibits RelB subunit nuclear translocation, suggesting that this subunit could be involved in PACAP-induced TNF down regulation [12]. RelB knockdown in PC12 cells showed that this subunit is indeed required for TNF constitutive expression and could be responsible for PACAP-induced TNF inhibition. These observations are in line with previous results obtained in immune cells showing that the RelB subunit of NF-κB is able to bind κB regulatory sites of the TNF gene promoter, and that the absence of this subunit in RelB-deficient animals results in a impaired production of TNF by macrophages in response to cytokines [21]. Furthermore, RelB-containing complexes participate in the constitutive κB activity observed in lymphoid tissues [22,23]. In macrophages, PACAP-induced inhibition of TNF expression has been ascribed to (i) reduction of IKK activity and NF-κB nuclear translocation and (ii) competition between NF-κB and PACAP-induced CREB for their common coactivator, the CRE-binding protein, leading to an overall decrease in NF-κB activity and TNF expression [24]. Similar mechanisms could occur in PC12 cells to mediate the effect of PACAP on TNF expression and the resultant action of the cytokine. Indeed, PACAP inhibits RelB nuclear translocation [12] and stimulates CREB activity in PC12 cells [25]. Interestingly, immunohistochemical analysis of sympathetic ganglia in mouse embryos revealed the expression of RelB in developing sympathetic neurons, suggesting that RelB could be involved in TNF expression in vivo. The production of the cytokine has previously been demonstrated in sympathetic neuroblasts, where it induced apoptosis [17].

Our data show that constitutive activity of the RelB subunit within NF-κB is required for constitutive expression of TNF in PC12 cells. The cytokine is secreted by PC12 cells and interacts with TNF-R1 to induce apoptotic cell death. Down-regulation of TNF gene expression by PACAP via RelB inhibition would be among the trophic effects of the neuropeptide, acting through inhibition of the apoptotic and anti-differentiation actions of the cytokine. These data suggest that TNF autocrine/paracrine effects in vivo could be implicated in the death of symapathetic neuroblasts that fail to obtain sufficient PACAP, as has been shown for nerve growth factor-dependent neurons during sympathetic neuron development [17].

Acknowledgments

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